1993

The effects of teratogens on serotonin expression in drosophila embryonic cell cultures

Keri Joel Hopkins

THE EFFECTS OF TERATOGENS ON SEROTONIN EXPRESSION IN DROSOPHILA EMBRYONIC CELL CULTURES

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

---

by
Keri Joel Hopkins
September 1993

THE EFFECTS OF TERATOGENS ON SEROTONIN EXPRESSION IN DROSOPHILA EMBRYONIC CELL CULTURES

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

---

by
Keri Joel Hopkins
September 1993

Approved by:

[redacted]

Dr. Nicole Bournias, Chair — 7-28-93 Date

[redacted]

Dr. Richard Fehn

[redacted]

Dr. Jeffrey Thompson

ABSTRACT

An *in vitro* teratogen assay has been developed utilizing *Drosophila* embryonic cell cultures and immunohistochemical techniques. It has previously been established that inhibition of differentiation, cell-to-cell communication and cell migration are all operating mechanisms of teratogenesis. The morphological endpoints used in assessing the teratogenic response of this assay involve indentifying the inhibition of neuron and/or muscle differentiation, induction of heat shock proteins, and inhibition of normal neurotransmitter levels. This project investigated the effects of suspected neuroteratogens on levels of serotonin expression in *Drosophila* embryonic cell cultures. The cultures were incubated for 18 hours in the agent to be tested and then processed using a standard ABC protocol. The cells are visualized by incubation in the peroxidase substrate diaminobenzidine (DAB) yielding a brown precipitate on the serotonin producing cells. A chemical is classified as eliciting a teratogenic response if it results in a statistically significant reduction in the number of positively stained neuronal clusters when compared to untreated controls. Results indicate that the *Drosophila* assay is capable of accurately establishing whether the particular agent tested can act as a teratogen by a variety of appropriate endpoints (morphological, biochemical, and molecular). It is hoped that this assay can be used not only as a teratogen screen, but also in mechanistic studies of abnormal development and gene involvement in teratogenic resistance.

ACKNOWLEDGEMENTS

I would like to thank the faculty and staff of the Biology Department at Cal State for their continued encouragement and support in seeking my graduate degree.

To my family and friends: To my Mom and Dad, thank you for your everlasting love and financial support through many years of school. To my mother- and father-in-law, thank you for your many hours and days of grandson sitting so that I could take those many needed breaks!

To my boss and thesis advisor, Dr. Bournias: Thank you for everything! I can't say enough for all the support and experience I have gotten from your lab and instruction, it will always be a memorable and special part of my life.

To my husband and son: Doug, thank you for being without a mommy many days and many evenings, I know someday you will understand why I did it. Know that I will always love you even when I'm not there to tuck you in at night. Greg, I love you! Thank you for your constant encouragement and belief in me. I especially want to thank you for being a wonderful daddy on the many occasions when I was not home or too tired to move. Most of all, thank you for understanding the importance of my education knowing that someday I will be done and it will all be worth it!

Thank you all, I couldn't have done it without any one of you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Teratology can be defined as the study of irreversible developmental anomalies of cell function and/or morphology. Populations of humans and animals are exposed more and more frequently to potentially teratogenic compounds in the form of commercial chemicals, industrial by-products, wastes, and a number of drugs which have not been properly tested. These agents can cause birth defects directly by interfering with embryonic development, or indirectly by gene mutation. Such teratogens are now estimated to cause approximately 4-5% of all human birth defects, though the actual percentage may be much higher due to minor deficiencies undetected at birth (Wilson, 1977). For example, several studies have recently been done on children exposed to alcohol *in utero*. They have been found to have problems ranging from learning and behavioral deficits to severe mental retardation (Zimmerberg et al., 1991; Boyd et al., 1991; Adickes et al., 1990; Conry, 1990; Nanson and Hiscock, 1990). The more severe cases can be detected at birth, however the minor deficiencies usually are not seen until early childhood.

One of the major objectives of teratology is to anticipate risks before they happen. Traditionally, *in vivo* assays were developed and used in testing suspected teratogens, but these involved exposure of embryos *in utero* and then classification of the abnormalities at birth. These *in vivo* assays proved to be expensive, time consuming and not always predictive due to the variation in species-specific responses.

As a result of the problems encountered with *in vivo* testing, and with 50,000-70,000 different chemicals already in the market place, and some 200-400 new ones produced every year, several alternative *in vitro* assays have recently been developed. These assays range from whole embryo cultures, derived from invertebrates and vertebrates, to cell and organ cultures (Neubert and Barrach, 1977). It is hoped that the successful development and

validation of several such assays will serve as prescreens for testing teratogens and provide additional information of their mechanisms, especially at the molecular level.

One such assay that was developed utilizes *Drosophila* embryonic cell cultures (Bournias-Vardiabasis et al., 1983). This assay is based on the principle that inhibition of differentiation, cell-to-cell communication and cell migration are all operating mechanisms of teratogenesis (Wilson,1977). The morphological endpoints used in assessing the teratogenic response of this assay involves identifying the inhibition of neuron and/or muscle differentiation.

The *Drosophila* system is ideal for the *in vitro* study of embryonic development because the culture develops in predictable steps leading to the differentiated cell state. Muscle and neuron differentiation show temporal, sequential, and morphological characteristics, which provide a suitable measure of differentiation for the evaluation of teratogens. In morphology studies, a tested compound is considered as eliciting a teratogenic response if it results in a significant reduction (>50%) in the number of myotubes and ganglia when compared to controls. Each chemical is tested in three different trials before a classification is assigned. These tests have resulted in the classification of over 150 compounds (Bournias-Vardiabasis and Flores, 1983; Bournias-Vardiabasis *et al.* 1983; Bournias-Vardiabasis, 1983; Bournias-Vardiabasis and Flores, 1986) with a low number of false positives and false negatives.

From testing and literature searches, it became apparent that there will be some teratogens which do not affect cellular morphology, but rather alter function or inhibit synthesis of enzymes necessary for the normal metabolism of various biochemicals, including neurotransmitters. Chemicals that may be particularly toxic to the developing neural tissue have been classified as neuroteratogens (Bournias-Vardiabasis, 1990). The efficacy of the *Drosophila* assay has been extended to include assessment of levels of neurotransmitters and the enzymes involved

in their synthesis and degradation. The successful development of an assay that correctly identifies neuroteratogens could be of tremendous aid since currently only postnatal behavioral assays exist for neural defects and a major drawback of such tests is they are not quantifiable. An alternative quantifiable assay, such as the *Drosophila* assay, would also help to detect the many subtle deficiencies which go undetected in neonatal tests but later become evident in early childhood.

Neurotransmitters have been reported to function in regulation of morphogenesis, growth, and differentiation in many different species (Schwartz, 1990; Sieber-Blum et al, 1983; Shuey et al., 1990; Jurand, 1980; Zimmerman, 1985; Huff et al., 1989). It has been suggested that monoamines may play a "nontransmitter" role since they are present during embryogenesis before development of the nervous system (Zimmerman and Wee, 1984). For example, serotonin and acetylcholine have been proposed to control cell division and cell movements during morphogenesis of the sea urchin. The role of serotonin appears to be that of regulation of the mesenchymal cell movement during gastrulation (Gustafson and Toneby, 1970).

In the chick embryo, catecholamines and serotonin have been shown to be concentrated in the notochord and developing neural tube (Kirby and Gilmore, 1972; Lawerence and Burden, 1973; Wallace, 1979). Perturbing neurotransmitter levels of serotonin in the chick embryo by disturbances in its metabolism caused teratogenic effects in neural tube closure showing its involvement in regulation of early morphogenetic movements (Palen, 1979).

Another role of serotonin in neurogenesis is that it may serve as a trophic agent in the development of target cells of serotonergic neurons. In embryonic rat brain, when serotonin synthesis is inhibited by maternal administration of *p*-chlorophenylalanine, the beginning of differentiation of certain neuronal populations is delayed (Lauder and Krebs, 1978). Lauder *et al.* showed with immunocytochemical techniques that these populations are derived

from regions of the neuroepithelium through which serotonergic axons are growing. They hypothesized that the serotonergic neurons may have a trophic influence on the proliferation and differentiation of their neuronal and glial target cells in the developing brain (Lauder *et al.*, 1982).

Neurotransmitters have also been well studied in insects including the grasshopper (Taghert and Goodman, 1984; Doe and Goodman, 1985) and the fruit fly *Drosophila* (Valles and White, 1988; Budnik and White, 1988; Huff *et al.*, 1989). Four major classes of putative neurotransmitters, or neuromodulators, have been identified in the *Drosophila* nervous system: (i) cholinergic, (ii) biogenic amines, (iii) amino acids, and (iv) neuropeptides. Most work has been done with acetylcholine therefore it appears to be the major transmitter system in the central nervous system of *Drosophila* (Restifo and White, 1989). Salvaterra *et al* have reported on the characteristics of *in vitro* neuronal differentiation of *Drosophila* embryonic cells, the temporal and spatial expression of choline acetyltransferase (ChAT) and acetylcholine esterase (AchE), and the effect that some cholinergic agents have on normal patterns of neurotransmitter expression (Salvaterra *et al.*, 1987).

The embryonic central nervous system of *Drosophila* and other insects arises from a group of neuroblasts which first become distinguishable shortly after gastrulation. At this time, the neuroblasts enlarge and segregate internally from the primitive ventral ectoderm. These cells then undergo a series of asymmetric cell divisions to produce ganglion mother cells (Poulson, 1950; Bate, 1976). Each ganglion mother cell divides once more to generate two daughter cells which differentiate as neurons. Each neuroblast is therefore capable of producing a family of neurons, or cell lineage, of variable size (Huff *et al*, 1989).

Huff *et al.* (1989) showed, using embryonic cell cultures, that both serotonin and dopamine were observed in neuron clusters derived from individual neuroblasts. There were one, two, or three neurons per cluster. These results implied that the neurons within

the cluster are clonally related, and that the differentiation steps for serotonin and dopamine synthesis are autonomous. Through lineage truncation experiments Huff *et al.* (1989) found that serotonergic cells are products of early cell divisions occurring between the seventh and eleventh hours of development which correspond to the first to the third neuroblast cell divisions.

The *Drosophila* cell culture technique is well established (Seecof, 1979), and neurogenesis and myogenesis are normal by all tests done to this date. The events *in vitro* match those reported *in vivo* and proceed at about the same rate with only a short time lag (Seecof et al., 1973; Dewhurst and Seecof, 1975). In these primary cultures, several cell types differentiate from their respective stem cells during a 24-hour period. Muscle and neuronal cells represent 70-80% of the cell population thus differentiation has been selected for the morphological endpoint of the assay. Neuroblasts, in cultures as well as *in vivo*, undergo a series of eight unequal divisions which begin shortly after the initiation of gastrulation. This is followed by a final round of division of the daughter cells, giving rise to clusters of about 18 neurons (Bournias-Vardiabasis *et al.*, 1983). Neuron differentiation *in vitro* and in the embryo, is asynchronous, the neuroblast divisions begin at about the onset of gastrulation at 3.5 hours and continue until about 17.5 hours after oviposition. The daughter neurons recognize and adhere to each other forming miniature ganglia with cell bodies at the periphery and a neuropile with synapses in the interior (Gerson et al., 1976). Short processes are observed first in the culture by eight hours after oviposition, and axons 50 microns or longer are present at 11 hours and increase in length and number for 20 hours (Donady et al., 1975). The neurotransmitter, serotonin, is first detected at 7 hours of development.

This project investigates the effects of suspected neuroteratogens on serotonin expression in *Drosophila* embryonic cell cultures. The cultures were scored for positive serotonin clusters and cell morphology. It was anticipated that exposure of

embryonic *Drosophila* cells to known or suspected neuroteratogens would result in a reduction in serotonin levels and the reduction would be dose dependent.

The tested chemicals (Table 1) are a subset chosen from a list published by the Consensus Workshop on In Vitro Teratogenesis Testing (Smith et al., 1983). This list was developed because investigators agreed that controlled test validation would require both standardization among laboratories with respect to the test compounds used, and agreement as to their teratogenicity or lack thereof. A panel selected compounds which, in the absence of maternal toxicity, gave rise to gross structural abnormalities, embryolethality, growth retardation, and/or perinatal and postnatal functional deficits *in vivo* (Smith *et al.*, 1983). The chemicals used in the *Drosophila* serotonin assay were chosen for their suspected effect on neurogenesis during development. Chemical doses were determined at 1% of the LD_{50} value established for that particular agent.

This project utilizes immunohistochemical protocols, specifically the "sandwich" technique, in which a primary antibody is followed by a biotinylated secondary antibody and the avidin-biotin HRP complex (ABC) to form an antibody "sandwich". This is followed by a peroxidase substrate solution resulting in the formation of a brown precipitate on the serotonin producing cells.

Compound	Concentration (mM)
Ethanol	1%
	3%
Mercuric Chloride	.01
Diphenylhydantoin	.05
Scopolomine	.01
Methyltestosterone	0.1
Chloroquine	0.5
Bromodeoxyuridine	1
Carbachol	.01
L-DOPA	1
Phenothiazine	.01
Progesterone	.01
Thalidomide	.01
Valproic Acid	1
Cortisone	1
Diazepam	0.1
Hyperthermia	42° for 20 min.

Table 1: List of teratogens tested in the serotonin assay. The teratogens listed are a subset of the list published by the Consensus Workshop on In Vitro Teratogenesis (Smith et al., 1983) and were chosen for their implications as putative neuroteratogens.

MATERIALS AND METHODS

Fly stocks. *Drosophila melanogaster* Canton S. strain were raised in population cages at 25°C with a 12 hour light/dark cycle.

Collections. *Drosophila* embryos were collected on plates of standard cornmeal medium supplemented with live yeast. Collections were for two hours then the embryos were allowed to develop at room temperature to the early gastrulation stage at which time all cells have been determined. After 5.5 hours, the embryos were gently removed from the plates with water and a soft brush. The embryos were then passed through a screen and collected on a fine mesh cloth.

Cell Culture. Embryos were dechorionated and surface sterilized in a 1:1 mixture of 95% ethanol and Clorox bleach for two minutes. They were then gently homogenized and the cells pelleted at 1000rpm for 4 minutes. The number of cells was estimated by standard hemocytometer methods and plated out in 35mm tissue culture dishes (Nunc) containing three round13mm glass coverslips. The final cell density per dish was 8 x 10^5 cells per 2ml of modified Schneider's medium supplemented with 18% fetal calf serum (Intergen). After the cells attached to the coverslips (15-20 minutes), the medium was removed and replaced with medium containing the compound to be tested. Control cultures received a change of medium lacking teratogen. The plates were allowed to incubate at room temperature for 18-24 hours.

Development. Developmental studies for serotonin expression were done on embryonic cell cultures. The cells were assayed at 5.5, 7.5, and 12 hours (after oviposition) of development. The percentage of positive-serotonin clusters remained the same as expected for *in vivo* development at equivalent times.

Immunohistochemical Assay. Treated cell cultures were washed with *Drosophila* saline and then phosphate buffered saline (PBS) containing 1 mg/mL bovine serum albumin (BSA). They were

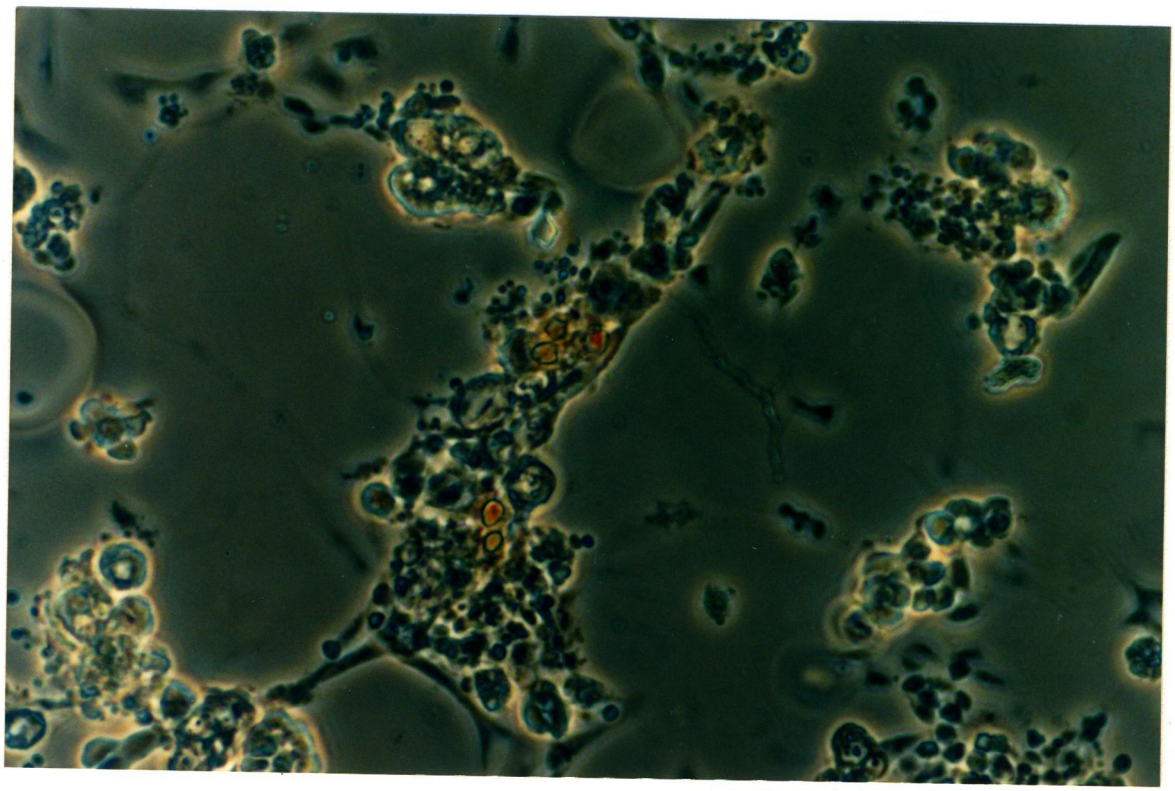
then fixed with 4% paraformaldehyde for 30 minutes. After the cells were rinsed with the PBS-BSA buffer, they were incubated in 3% goat serum (Vectastain ABC Kit) to block non-specific antibody binding. Cells were then incubated with rabbit anti-serotonin antibody (Incstar) at a dilution of 1:200 in PBS, 1% BSA and 0.1% Triton-X 100 for two hours at room temperature in a humidified chamber. The primary antibody was applied using the hanging drop method to conserve amounts of antibody used in each trial. This was followed by incubation in biotinylated anti-rabbit IgG (Vectastain ABC Kit) at a dilution of 1:200 in PBS-BSA for one hour at room temperature. The cells were then incubated in the avidin-biotin horseradish peroxidase complex (ABC) reagent (Vectastain ABC Kit) for one hour followed by the color reaction. The chromagen used was 0.05% diaminobenzadine tetrahydrochloride (DAB), 0.01% H_2O_2 in 0.1M Tris buffer. Incubation in the peroxidase substrate for 3-5 minutes yielded a brown precipitate on the serotonin producing cells (Figure 1).

Scoring. The affect of each treatment was determined in at least three separate experiments run on different days. In each experiment, the number of neuronal clusters were counted and the number of serotonin positive clusters noted. Three control and three experimental coverslips were scored in each experiment. Ten fields per coverslip were scored and the average percentage of serotonin positive clusters determined. This design resulted in at least three separate estimates of the percentage of responding clusters per experiment.

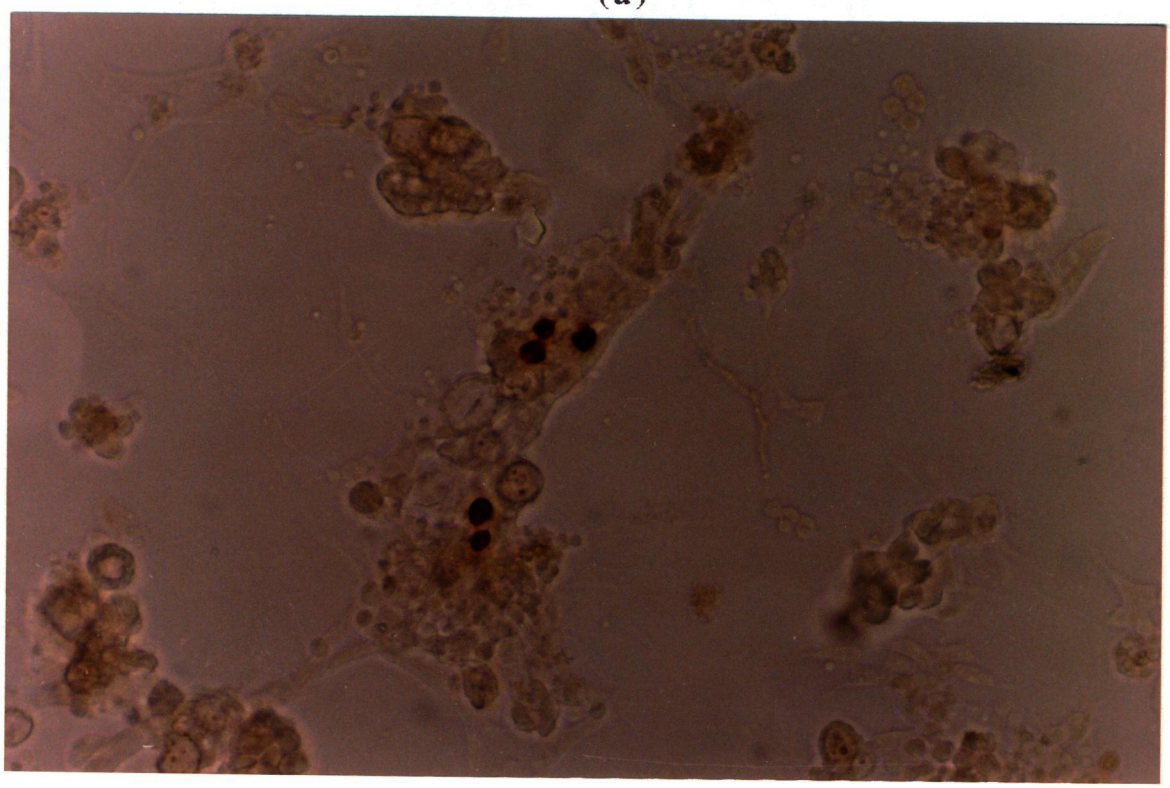
Data Analysis. Two types of analysis were done. First, the percentage of positive responding clusters of each experiment was compared to the percentage of positive responding clusters in the control of that experiment. The results of the three teratogen trials were averaged as were the controls for those trials, and the percentage of control calculated. Additional analyses were done using Analysis of Variance. The affect of treatment was determined using a randomized complete block design, with blocks being

experiments performed on different days. Within each block the mean percentage of serotonin positive clusters on different coverslips were treated as replicate observations. A separate ANOVA was run for each compound tested using the General Linear Models (GLM) procedure of the SAS statistical analysis system (SAS Institute, 1985).

Figure 1: Photomicrograph of a 20-hour differentiated *Drosophila* embryonic cell culture after ABC assay. The cells of the ganglia expressing serotonin are stained brown. a) phase-contrast b) bright field.



(a)



(b)

RESULTS

The developmental studies of serotonin expression showed that at 5.5 hours after oviposition there was no cell type-specific morphology and the cells appeared round and undifferentiated. At this time there was some serotonin expression with approximately one out of ten fields containing a positive serotonin cell. After 7.5 hours of development, there was still little differentiation, but small clusters of 3-5 cells were visible and serotonin expression had increased about two-fold. At twelve hours of development differentiated cell types were easily recognizable. Many of the cells were arranged in clusters which contained fine cell processes extending outward from the cluster. At this stage another cell type, myocytes, were present in the culture, fusing to form the multinucleated myotubes. Serotonin expression at this time had reached its full expression. By 17 or 18 hours of development, most of the myocytes had fused to form the multinucleate myotubes with 3-5 nuclei. The neuronal-type cells were present primarily in clusters with extensive process development. The amount of serotonin expression remained constant in the culture.

The influence of the tested teratogens on serotonin expression in treated *Drosophila* embryonic cultures are summarized in Table 2. All but three of the compounds tested (methyl testosterone, phenothiazine, and progesterone) caused a significant decrease in serotonin expression as compared to the control cultures. The compounds cortisone and thalidomide elicited the greatest reduction in serotonin expression at 19% and 0.3% of controls, respectively.

A large number of compounds have already been tested with the *Drosophila* morphology assay (Bournias-Vardiabasis et al., 1983), with a small number of false positives (non-teratogens that test positive) and false negatives (drugs that have been assessed as acting as teratogens in a variety of animal assays that test negative in this assay). Table 3 shows the comparison of results between the

Drosophila morphological endpoint assay and the *Drosophila* serotonin assay. In several cases, i.e. ethanol, methyl testosterone, phenothiazine, progesterone, L-DOPA, and cortisone, the assay results show different teratogen classifications.

In order to support and verify the results of the serotonin assay, a statistical analysis was also done with the raw data generated from the experiments. Table 4 shows a comparison of the results of the two types of analyses; the resulting P- values correspond in significance to the percent of control analysis method shown previously.

Two teratogens were studied in more detail at varying concentrations to examine the response of serotonin expression. These studies were done to demonstrate that the assay is sensitive enough to detect minor changes in serotonin expression which parallel differences in drug dosage. The results of these tests are summarized in Figure 2. With increasing concentration of teratogen, serotonin expression levels decreased.

Compound	Concentration	% serotonin '+' clusters
Ethanol	1%	49
	3%	31
Mercuric Chloride	.01	66
Diphenylhydantoin	.05	75
Scopolomine	.01	70
Methyltestosterone	0.1	82
Chloroquine	0.5	42
Bromodeoxyuridine	1	73
Carbachol	.01	75
L-DOPA	1	62
Phenothiazine	.01	88
Progesterone	.01	79
Thalidomide	.01	0.3
Valproic Acid	1	38
Cortisone	1	19
Diazepam	0.1	25
Hyperthermia	42° for 20 min.	90

Table 2: Effects of teratogens on serotonin synthesis in *Drosophila* embryonic cell cultures. The following 17 compounds and/or treatments were tested on *Drosophila* embryonic cell cultures to examine their effects on serotonin expression. The number of serotonin positive clusters were compared to the number in the corresponding controls and a percentage value was calculated. A positive effect, i.e. eliciting a significant decrease in expression, for the molecular endpoint assay was taken as less than or equal to 75% of the control.

Compound	Drosophila Morphology Assay	Drosophila Serotonin Assay
Ethanol	-	+
Mercuric Chloride	+	+
Diphenylhydantoin	+	+
Scopolomine	ND	+
Methyltestosterone	+	-
Chloroquine	+	+
Bromodeoxyuridine	+	+
Carbachol	ND	+
L-DOPA	-	+
Phenothiazine	+	-
Progesterone	-	-
Thalidomide	+	+
Valproic Acid	ND	+
Cortisone	-	+
Diazepam	+	+
Hyperthermia	-	-

[a]The designation + or - refers to comparison of the neuron and/or muscle count to controls; + indicates a significant decrease in the neuron and/or muscle count; - indicates an insignificant decrease in the neuron and/or muscle count; ND = not determined.

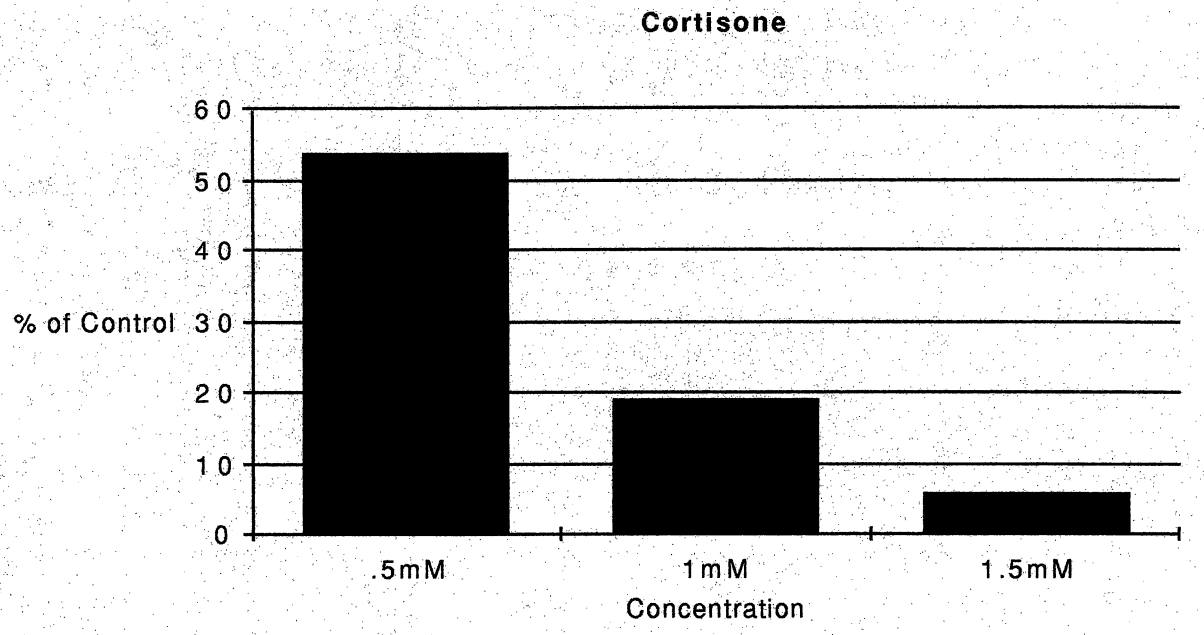
Table 3: Comparison of the results from the *Drosophila* serotonin assay and the *Drosophila* morphology assay.

Compound	% serotonin '+' clusters	P value	Drosophila Serotonin Assay
1% Ethanol	49	0.0002***	+
3% Ethanol	31	0.0001***	+
Mercuric Chloride	66	0.0036**	+
Diphenylhydantoin	75	0.0001****	+
Scopolomine	70	0.0054**	+
Methyltestosterone	82	0.1291	-
Chloroquine	42	0.0001***	+
Bromodeoxyuridine	73	0.0023**	+
Carbachol	75	0.0246*	+
L-DOPA	62	0.0104*	+
Phenothiazine	88	0.1184	-
Progesterone	79	0.0572	-
Thalidomide	0.3	0.0001***	+
Valproic Acid	38	0.0001***	+
Cortisone	19	0.0007***	+
Diazepam	25	0.0001***	+
Hyperthermia	90	0.2120	-

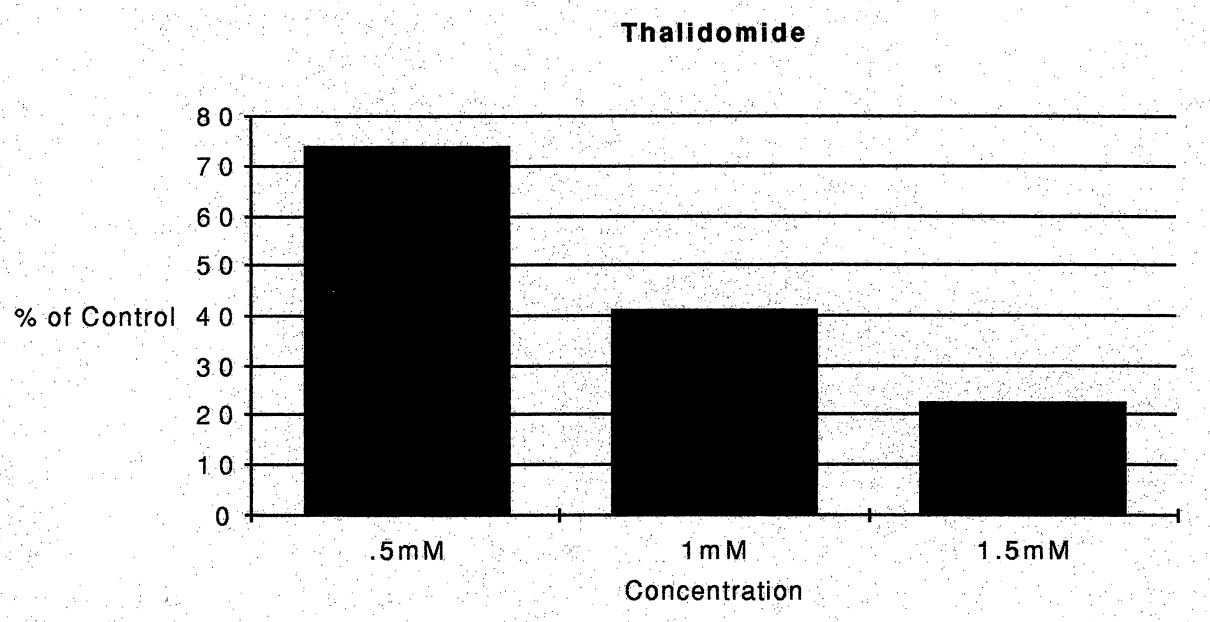
* $p < .05$
** $p < .01$
*** $p < .001$

Table 4: Comparison of analysis methods. The data was analyzed by percent of control (see Materials and Methods) and then statistical analysis for verification of the results.

Fig 2: Histograms showing dose-response of serotonin expression when treated with varying concentrations of (a) cortisone, or (b) thalidomide.


Cortisone
60
50
40
% of Control 30
20
10
0
.5mM
1mM
1.5mM
Concentration

(a)


Thalidomide
80
70
60
50
% of Control 40
30
20
10
0
.5mM
1mM
1.5mM
Concentration

(b)

DISCUSSION

Evidence suggesting a role for neurotransmitters in directing normal embryonic development is rapidly accumulating. The neurotransmitter serotonin has been implicated in several roles during development: as a growth factor (Seuwen and Pouyssegur, 1990), in neural crest cell differentiation (Seiber-Blum et al., 1983), in palate development (Zimmerman, 1985), in morphogenesis (Shuey et al., 1990; Zimmerman, 1988), and as a neurotrophic factor (Schwartz, 1990). These developmental roles were determined by the application of specific drugs to cultures which led to interference with serotonin synthesis, serotonin release and receptor interactions.

The *in vitro Drosophila* assay has shown a high degree of concordance between agents assessed as teratogenic in the *Drosophila* system and in other systems, such as *in vivo* animal studies and human epidemiological studies (Table 5). Because the assay monitors cell death and proliferation events, the results indicate that measurement of neurotransmitters at a final differentiated cellular state is an efficacious criterion for teratogenicity evaluation. Interference with any preceding step in the differentiation process, including cell viability and rate of proliferation, will affect the outcome. That is, if a treated cell does not differentiate and survive, or if it does not differentiate in time for determination, serotonin will not be produced by that cell which could be seen in the assay.

The results of the *Drosophila* serotonin assay classified twelve out of seventeen tested compounds as neuroteratogens, that is, they elicited a decrease in serotonin expression. The mechanism of this decrease in expression has not been determined, however it is hoped that more details of the mechanistic pathway will be evident as new experimental results are reported.

Test results also revealed differences in the compound classifications between the *Drosophila* morphology assay and the *Drosophila* serotonin assay. These results are significant in that they show that one assay cannot reveal the total effects of teratogen exposure. The different structures and functions of various compounds will allow them to affect some processes (e.g. morphology) and not others (e.g. neurotransmitter expression). It is for this reason that a panel of assays must be developed to accurately evaluate the effects of teratogens. A key example of this need is that of ethanol exposure. In the assays, ethanol showed negative results in the morphology assay but highly significant positive results in the neurotransmitter assay indicating different mechanistic pathways. This neuroteratogen has been heavily researched in the past few years in relation to fetal alcohol syndrome and neuropsychological deficits (Conry, 1990; Boyd et al., 1991; Nanson and Hiscock, 1990).

The *Drosophila* assay can potentially be used as a Tier I screen in a clinical setting as well as a tool for understanding mechanisms of teratogenesis. The vast amount of biochemical, molecular, and developmental knowledge of *Drosophila* should provide important clues as to the roles of genetic and biochemical variables in the process of teratogenesis. Comparison of the results from the serotonin neurotransmitter assay with those of the ChAT enzyme assay (Bournias-Vardiabasis, unpublished results) in Table 6, show that there may be intra-assay differences in the classification of a teratogen too, depending on which class (in this case, indolemine vs. catecholemine) of neurotransmitter is being tested. Two of the teratogens tested in these assays, methyltestosterone and phenothiazine, were determined as non-neuroteratogens in the serotonin assay, but were positive neuroteratogens in the ChAT assay. Examples of the reverse can be seen with cortisone and diazepam. The differences between the morphological and molecular endpoint studies, as well as differences within the molecular endpoint studies, again emphasize that an array of assays must be

developed to determine the holistic effects of exposure to teratogenic compounds.

Currently, similar work is being done with the neurotransmitter histamine. The data from this project, along with the information gained from the serotonin and acetylcholine projects, will demonstrate the effects of teratogens on different classes of neurotransmitters. However, this is only one level of many needed for effective screening of effects of teratogen exposure.

Future studies that would be informative in the mechanism of teratogenesis include double labeling experiments in which two neurotransmitters could be assessed simultaneously to observe changes in expression levels. If expression of one neurotransmitter is decreased in a cell population, is another increased to compensate for the deficiency? If so, what effects does this have on the cell population? Another study on effects of teratogen exposure on cell cultures could be done in which it is determined whether exposure to a teratogen results in the turning on or off of a particular gene, or just modification of expression of that gene leading to the particular characteristic phenotype. Lastly, an experiment could be designed to determine whether the teratogen is having its affect before or during differentiation. This could be accomplished by incubating the cells in serum minus medium plus teratogen, i.e. exposing the cells to the chemical in the G phase of the cell cycle. After a determined time, remove the teratogen and add medium with serum and allow the cells to differentiate then assay for neurotransmitter expression. Are expression levels comparable before and after differentiation?

The importance for developing such an array of assays is becoming more apparent everyday as new research shows that fetuses exposed to teratogenic agents develop a range of disabilities from severe mental retardation to learning and behavioral deficiencies. It is these minor deficiencies, which are not phenotypically expressed at birth and thus go unnoticed until

problems arise during young adolescence, that are of great interest and would greatly benefit from such a screen of assays. But more importantly, public awareness of the risks of exposure to potentially harmful compounds during pregnancy may be our most effective weapon against the occurrence of such unnecessary tragedies.

Compound	Human Morphology Assay	Animal Morphology Assay	Drosophila Morphology Assay	Drosophila Serotonin Assay
Ethanol	+[a]	+	-	+
Mercuric Chloride	+	+	+	+
Diphenylhydantoin	+	+	+	+
Scopolomine	+	+	ND	+
Methyltestosterone	+	+	+	-
Chloroquine	+	+	+	+
Bromodeoxyuridine	ND	+	+	+
Carbachol	ND	+	ND	+
L-DOPA	ND	+	-	+
Phenothiazine	+	+	+	-
Progesterone	+	-	-	-
Thalidomide	+	+	+	+
Valproic Acid	+	+	ND	+
Cortisone	+	+	-	+
Diazepam	S	+	+	+
Hyperthermia	S	+	-	-

[a]The designation + or - refers to comparison of the neuron and/or muscle count to controls; + indicates a significant decrease in the neuron and/or muscle count; - indicates an insignificant decrease in the neuron and/or muscle count; S = suspect; ND = not determined.

Table 5: Comparison of *Drosophila* morphology and serotonin assay results to classifications assigned by Smith et al. (1983) from *in vitro* validation tests.

Compound	Concentration	% serotonin '+' clusters	% ChAT Activity
Ethanol	1%	49	34
	3%	31	ND
Mercury Chloride	.01	66	18
Diphenylhydantoin	.05	75	54
Scopolomine	.01	70	50
Methyl Testosterone	0.1	82	54
Chloroquine	0.5	42	17
Bromodeoxyuridine	1	73	27
Carbachol	.01	75	50
L-DOPA	1	62	15
Phenothiazine	.01	88	51
Progesterone	.01	79	88
Thalidomide	.01	0.3	45
Valproic Acid	1	38	13
Cortisone	1	19	150
Diazepam	0.1	25	126
Hyperthermia	42° for 20 min.	90	ND

ND = not determined

Table 6: Comparison of results of the *Drosophila* serotonin neurotransmitter assay and the *Drosophila* ChAT enzyme assay.

REFERENCES

Adickes, E.D., T.J. Mollner and S.K. Lockwood (1990). Ethanol induced morphologic alterations during growth and maturation of cardiac myocytes. *Alcohol Clin Exp Res.* 14(6), 827-831.

Bate, C.M. (1976). Embryogenesis of an insect nervous system I. A map of the thoracic and abdominal neuroblasts in *Locusta migratoria.* *J. Exp. Morphol.* 35(1), 107-123.

Bournias-Vardiabasis, N. (1983). On the teratogenic effect of coumarin in *Drosophila melanogaster.* *Drosophila Information Service* 59, 24-25.

Bournias-Vardiabasis, N. and J. Flores (1983). Drug metabolizing enzymes in *Drosophila melanogaster*: teratogenicity of cyclophosphamide *in vitro.* *Teratogenesis, Carcinogenesis and Mutagenesis* 3, 255-262.

Bournias-Vardiabasis, N., C.H. Buzin, and J.G. Reilly (1983). The effect of 5-azacytidine and cytidine analogs on *Drosophila melanogaster* cells in culture. *Wilhelm Roux's Archives of Developmental Biology* 192, 299-302.

Bournias-Vardiabasis, N. and J. Flores (1986). Response of *Drosophila* embryonic cells to tumor promoters. *Toxicology and Applied Pharmacology* 85, 196-206.

Bournias-Vardiabasis, N. (1990) *Drosophila melanogaster* embryo cultures: An *in vitro* teratogen assay. *ATLA* 18, 291-300.

Boyd, T.A., C.B. Ernhart, T.H. Greene, R.J. Sokol and S. Martier (1991). Prenatal alcohol exposure and sustained attention in the preschool years. *Neurotoxicology and Teratology* 13(1): 49-56.

Budnick, V. and K. White (1988). Catecholamine containing neurons in *Drosophila melanogaster*: Distribution and development. *J. Comp. Neurol.* 268, 400-413.

Conry, J. (1990). Neuropsychological deficits in fetal alcohol syndrome and fetal alcohol effects. *Alcohol Clin Exp Res.* 14(5), 650-655.

Craig, E.A. (1983). The heat shock response. *Critical Reviews of Biochemistry* 18, 239-280.

Dewhurst, S.A. and R.C. Seecof (1975). Development of acetylcholine metabolizing enzymes in *Drosophila* embryos and in cultures of embryonic *Drosophila* cells. *Comp. Biochem. Physiol.* 50C, 53-58.

Doe, C. and C. Goodman (1985). Early events in insect neurogenesis: the role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Dev. Biol.* 111, 206-219.

Donady, J.J., R.L. Seecof and S. Dewhurst (1975). Actinomycin D sensitive periods in the differentiation of *Drosophila* neurons and muscle cells *in vitro*. *Differentiation* 4, 9-14.

Gerson, I., R.L. Seecof, and R.L. Teplitz (1976). Ultrastructural differentiation during neurogenesis *in vitro*. *J. Neurobiol.* 7, 447-455.

Gustafson, T. and M. Toneby (1970). On the role of serotonin and acetylcholine in sea urchin morphogenesis. *Exp. Cell Res.* 62, 102-117.

Huff, R. , A. Furst, and A.P. Mahowald (1989). *Drosophila* embryonic neuroblasts in culture: autonomous differentiation of neural specific neurotransmitters. *Dev. Biol.* 134, 146-157.

Kirby, M.L. and S.A. Gilmore (1972). A fluorescence study on the ability of the notochord to synthesize and store catecholamines in early chick embryos. *Anat. Rec.* 173, 469-478.

Lauder, J.M. and H. Krebs (1978). Serotonin as a differentiation signal in early neurogenesis. *Dev. Neurosci.* 1, 15-30.

Lauder, J.M., J.A. Wallace and P. Petrusz (1982). Serotonin immunocytochemistry in the adult and developing rat brain: Methodological and pharmcological considerations. *Brain Res. Bull.* 9, 117-130.

Lawerence, I.E. and H.W. Burden (1973). Catecholamines and morphogenesis of the chick neural tube and notochord. *Am. J. Anat.* 137, 199-207.

Lindquist, S. (1986). The heat shock response. *Annu. Rev. of Biochem.* 55, 1151-1191.

Lindquist, S. and E.A. Craig (1988). The heat shock proteins. *Annu. Rev. of Genetics* 22, 631-677.

Nanson, J.L. and M. Hiscock (1990). Attention deficits in children exposed to alcohol prenatally. *Alcohol Clin Exp Res.* 14(5), 650-655.

Neubert, D., and H.J. Barrach (1977). Significance of *in vitro* techniques for the evaluation of embryotoxic effects. In *Methods in Prenatal Toxicology.* D. Neubert, H.J. Merker, and T.E. Kwasigroch, eds. Gerog Thieme, Stuttgart, pp. 202-209.

Palen, K., L. Thorneby and H. Emanuelsson (1979). Effects of serotonin and serotonin antagonists on chick embryogenesis. *Wilhelm Roux Arch. Dev. Biol.* 187(1), 89-103.

Poulson, D. (1950). Histogenesis, organogenesis and differentiation in the embryo of *Drosophila melanogaster.* In *Biology of Drosophila* (M. Demered, Ed.) pp. 168-274. Wiley, New York.

Restifo, L.L. and K. White (1989). Molecular and genetic approaches to neurotransmitter and neuromodulator systems in *Drosophila*. *Adv. in Inst. Phys.* 22, 64-65.

Salvaterra, P.M., N. Bournias-Vardiabasis, T. Nair, G. Hou and C. Lieu (1987). *In vitro* neuronal differentiation of *Drosophila* embryo cells. *J. of Neurosci.* 7(1), 10-22.

SAS Institute (1985). SAS User's Guide: Statistics Version 5 Edition. Cary, N.C.

Schwartz, J.P. (1990). Neurotransmitters as neurotrophic factors: A new set of functions. *International Review of Neurobiology* 34, 1-23.

Seecof, R.L., I. Gerson, J.J. Donady, and R. L. Teplitz (1973a). *Drosophila* myogenesis *in vitro*: the genesis of small myocytes and myotubes. *Dev. Biol.* 35, 250-261.

Seecof, R.L., J.J. Donady and R.L. Teplitz (1973b). Differentiation of *Drosophila* neuroblasts to form ganglion-like clusters of neurons *in vitro*. *Cell Differ.* 2, 143-419.

Seecof, R.L. (1979). Mass cultures of *Drosophila* embryonic cell cultures. *Tissue Culture Assoc. Manual.* 5, 1019-1022.

Seuwen, K. and J. Pouyssegur (1990). Serotonin as a growth factor. *Biochemical Phar.* 39, 985-990.

Shepard, T.H. (1981). Catalog of Teratogenic Agents. Johns Hopkins Press, Baltimore, MD

Shields, G. and J.H. Sang (1970). Characteristics of five cell types appearing during *in vitro* culture of embryonic material from *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* 23, 53-69.

Shields, G., A. Dubendorfer, and J.H. Sang (1975). Differentiation *in vitro* of larval cell types from early embryonic cells of *Drosophila melanogaster*. *J. Embryo. Exp. Morphol.* 33, 159-175.

Shuey, D.L., M. Yavarone, T.W. Sadler and J.M. Lauder (1990). Serotonin and morphogenesis in the cultured mouse embryo. In *Molecular Aspects of Development and Aging of the Nervous System* (ed. J.M. Lauder) Plenum Press, New York.

Sieber-Blum, M., W. Reed and H. Lidov (1983). Serotonergic differentiation of quail neural crest cells *in vitro*. *Dev. Biol.* 99, 352-359.

Smith, M.K., G.L. Kimmel, D.M. Kochhar, T.H. Shephard, S.P. Spielberg and T.G. Wilson (1983). A selection of candidate compounds for *in vitro* teratogenesis test validation. *Teratogenesis, Carcinogenesis, and Mutagenesis* 3, 461-480.

Taghert, P. and C. Goodman (1984). Cell determination and differentiation of identified serotonin-immunoreactive neurons in the grasshopper embryo. *J. Neurosci.* 4(4), 989-1000.

Valles, A. and K. White (1988). Serotonin-containing neurons in *Drosophila melanogaster*: Development and distribution. *J. Comp. Neurol.* 268, 414-428.

Wallace, J.A. (1979). Ph.D. thesis. University of California, Davis.

Wilson, J.G. (1977). Current status of teratology. In *Handbook of Teratology* (ed. J.G. Wilson and F.C. Fraser), p. 309. New York: Plenum Press.

Zimmerberg, B., H.L. Sukel, and J.D. Stekler (1991). Spatial learning of adult rats with fetal alcohol exposure: Deficits are sex-dependent. *Behav. Brain Res.* (42)1, 49-56.

Zimmerman, E.F. and E.L. Wee (1984). Role of neurotransmitters in palate development. In *Current Topics in Developmental Biology*. pp 37-63.

Zimmerman, E.F. (1985). Role of neurotransmitters in palate development and teratologic implications. In *Developmental Mechanisms: Normal and Abnormal* (ed. E.F. Zimmerman), pp. 283-294. New York: A.R. Liss.